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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US00/07050 <b>(22) International Filing Date:</b> 16 March 2000 (16.03.2000) <b>(30) Priority Data:</b> 09/276,868 26 March 1999 (26.03.1999) US <b>(60) Parent Application or Grant</b> BETH ISRAEL DEACONESS MEDICAL CENTER [/]; ( ). SIMONS, Michael [/]; ( ). GAO, Youhe [/]; ( ). PRASHKER, David ; ( ).		<b>Published</b>
<b>(54) Title: METHOD FOR PR-39 PEPTIDE REGULATED STIMULATION OF ANGIOGENESIS</b> <b>(54) Titre: METHODE DE STIMULATION DE L'ANGIOGENESE REGULEE PAR PEPTIDES PR-39</b>  <b>(57) Abstract</b> <p>The present invention provides both a method and means for regulating angiogenesis within living cells, tissues, and organs in-situ. The regulation is performed using native PR-39 peptide or one of its shorter-length homolog, for interaction with such proteasomes as one present in the cytoplasm of viable cells. The result of PR-39 peptide interaction with proteasomes is a decrease in the intracellular degradation of active peptides such as HIF-1'alpha' and a consequential stimulation of angiogenesis in-situ.</p> <b>(57) Abrégé</b> <p>La présente invention concerne à la fois une méthode et un moyen de régulation de l'angiogénèse dans des cellules vivantes, des tissus ainsi que des organes in situ. La régulation est exécutée à l'aide d'un peptide PR-39 natif ou d'un de ses homologues de longueur plus courte, afin d'avoir une interaction avec des protéasomes tels que ceux présents dans le cytoplasme de cellules viables. Le résultat de l'interaction du peptide PR-39 avec les protéasomes est une diminution de la dégradation intracellulaire de peptides actifs tels que HIF-1'alpha' et par conséquent une stimulation de l'angiogénèse in situ.</p>		

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(21) International Application Number: PCT/US00/07050 (22) International Filing Date: 16 March 2000 (16.03.00) (30) Priority Data: 09/276,868 26 March 1999 (26.03.99) US (71) Applicant: BETH ISRAEL DEACONESS MEDICAL CENTER [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US). (72) Inventors: SIMONS, Michael; 115 Grove Street, Chestnut Hill, MA 02467 (US). GAO, Youhe; Apartment 2, 85 Strathmore Road, Brighton, MA 02135 (US). (74) Agent: PRASHKER, David; David Prashker, P.C., 8 Chateau Heights, Magnolia, MA 01930 (US).		(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).  Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(54) Title: METHOD FOR PR-39 PEPTIDE REGULATED STIMULATION OF ANGIOGENESIS			
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**Description**

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METHOD FOR PR-39 PEPTIDE REGULATED STIMULATION  
OF ANGIOGENESIS

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CROSS REFERENCE

The present application is a Continuation-In-Part of prior pending United States Patent Application Serial No. 09/276,868 filed March 26, 1999.

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FIELD OF THE INVENTION

The present invention is concerned generally with the induction of angiogenesis within viable cells comprising living tissues and organs; and is particularly directed to mechanisms regulated by PR-39 peptides which result in a stimulation of angiogenesis on-demand and may be used as a controlled therapeutic treatment.

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BACKGROUND OF THE INVENTION

Angiogenesis, by definition, is the formation of new capillaries and blood vessels within living tissues; and is a complex process first recognized in studies of wound healing and then within investigations of experimental tumors. Angiogenesis is thus a dynamic process which involves extracellular matrix remodeling, endothelial cell migration and proliferation, and functional maturation

5 of endothelial cells into mature blood vessels [Brier, G. and K. Alitalo, Trends  
10 Cell Biology 6: 454-456 (1996)]. Clearly, in normal living subjects, the process of  
angiogenesis is a normal host response to injury; and as such, is an integral part of  
the host body's homeostatic mechanisms.

5 It will be noted and appreciated, however, that whereas angiogenesis  
represents an important component part of tissue response to ischemia, or tissue  
15 wounding, or tumor-initiated neovascularization, relatively little new blood vessel  
formation or growth takes place in most living tissues and organs of mature adults  
(such as the myocardium of the living heart) [Folkman, J. and Y. Shing, J. Biol.  
20 Chem. 267: 10931-10934 (1992); Folkman, J., Nat. Med. 1: 27-31 (1995); Ware,  
J.A. and M. Simons, Nature Med. 3: 158-164 (1997)]. Moreover, although  
regulation of an angiogenic response in-vivo is a critical part of normal and  
pathological homeostasis, relatively little is presently known about the control  
25 mechanisms for this process.

25 Overall, a number of different proteins, growth factors and growth factor  
receptors have been found to be involved in the process of stimulation and  
maintenance of angiogenic responses. For example, a number of cell membrane-  
30 associated proteins are thought to be involved in the processes of angiogenesis.  
Such proteins include SPARC [Sage et al., J. Cell Biol. 109: 341-356 (1989);  
20 Motamed K. and E.H. Sage, Kidney Int. 51: 1383-1387 (1997)]; thrombospondin  
1 and 2 respectively [Folkman, J., Nat. Med. 1: 27-31 (1995); Kyriakides et al.,  
35 J. Cell Biol. 140: 419-430 (1998)]; and integrins  $\alpha v \beta 5$  and  $\alpha v \beta 3$  [Brooks et al.,  
Science 264: 569-571 (1994); Friedlander et al., Science 270: 1500-1502 (1995)].  
In addition, a major role is played by heparin-binding growth factors such as basic  
40 25 fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF);  
and thus the regulation of angiogenesis is believed today to involve matrix  
components such as extracellular heparin sulfate and core proteins such as  
syndecans which are found at the surface of endothelial cells.

45 However, while a number of heparin binding growth factors (including  
30 VEGF, FGF1 and FGF2) have been shown to promote angiogenesis in-vitro and  
in-vivo, their process involvement appears limited to tissues demonstrating some  
50 form of inflammatory response to trauma (as defined by the presence of blood-

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5 derived macrophages), be it a direct tissue injury (such as wounding) or ischemia. Moreover, the presence of blood-derived macrophages is also routinely associated with localized secretion of a number of proteins including cytokines such as IL-2  
10 and TNF- $\alpha$ , growth factors such as VEGF and FGF-2, matrix metalloproteinases  
5 as well as many other biologically active molecules. Accordingly, although there have been many investigations, publications, and developments of these entities, there remains a general ignorance and failure of understanding by research  
15 investigators and clinicians alike regarding useful and effective specific means and methods for inducing angiogenesis on-demand within living cells, tissues, and  
10 organs. Thus, while the value and desirability of initiating new vascularization - especially using cells in localized areas on an as needed basis as well as a  
20 therapeutic treatment for individual patients - is well recognized, these aims remain a long sought goal yet to be achieved in a practical manner.

25 15 SUMMARY OF THE INVENTION

30 The present invention has multiple aspects and uses. A first aspect provides a method for stimulating angiogenesis within a targeted collection of viable cells in-situ, said method comprising the steps of:

20 identifying a collection of cells comprising viable cells in-situ as a target for stimulation of angiogenesis;

35 providing means for effecting an introduction of at least one member selected from the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells;

40 25 introducing at least one member of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells using said effecting means;

45 allowing said introduced PR-39 oligopeptide collective member to interact with such proteasomes as are present within the cytoplasm of said targeted collection of cells whereby

30 (a) at least some of the proteasomes interact with said PR-39 oligopeptide collective member, and

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(b) at least a part of the proteolytic activity mediated by said interacting proteasomes becomes selectively altered, and

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(c) the selectively altered proteolytic activity of said interacting proteasomes results in a stimulation of angiogenesis in-situ within the targeted

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collection of viable cells.

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A second aspect of the invention provides a method for selective inhibition of proteasome-mediated degradation of peptides in-situ within a collection of viable cells, said method comprising the steps of:

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identifying a collection of cells comprising viable cells in-situ as a target;

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providing means for effecting an introduction of at least one member

selected from the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells;

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introducing at least one member of the PR-39 oligopeptide collective to the

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cytoplasm of said targeted collection of cells using said effecting means;

allowing said introduced PR-39 oligopeptide collective member to interact

with such proteasomes as are present within the cytoplasm of said targeted collection of cells whereby

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(a) at least some of the proteasome interacts with the PR-39

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oligopeptide collective member, and

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(b) at least a part of the proteolytic activity mediated by said interacting proteasomes becomes markedly altered, and

(c) the markedly altered proteolytic activity of said interacting

proteasomes results in a selective inhibition of proteasome-mediated degradation of peptides in-situ within the targeted collection of cells.

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#### BRIEF DESCRIPTION OF THE FIGURES

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The present invention may be more fully understood and better appreciated

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when taken in conjunction with the accompanying drawing, in which

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Figs. 1A-1D are presentations of empirical data showing the direct interaction between PR-39 peptide and the  $\alpha 7$  subunit of proteasomes intracellularly;

Figs. 2A-2D are presentations of empirical data showing the effect of PR-39 peptide upon proteasome activity in-vivo;

Figs. 3A-3D are graphs demonstrating the results of in-vitro proteasome activity assays;

Figs. 4A-4C are presentations of empirical data showing the in-vivo effects of PR-39 peptide expression;

Figs. 5A-5C are photographs of representative sections showing differences in vascularity among control, PR-39 peptide and FGF2 impregnated Matrigel pellets;

Fig. 6 is a graph providing a quantitative analysis of vascularity for the representative sections of Fig. 5; and

Fig. 7 is a graph showing the induction of angiogenesis in-vivo using PR-39 peptide and short-length PR11 peptide impregnated Matrigel pellets.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is a method for stimulating angiogenesis via the purposeful introduction of native PR-39 peptide or a member of the PR-39 derived oligopeptide family to the cytoplasm of viable cells in-situ. The PR-39 peptide or the derived member of the family will interact with such proteasomes as are present intracellularly; and the consequence of PR-39 peptide/proteasome interaction is the selective inactivation of proteasomes such that intracellular degradation of proteins such as HIF-1 $\alpha$  and I $\kappa$ B $\alpha$  is diminished and a marked stimulation of angiogenesis in-situ consequently results.

A number of major benefits and advantages are therefore provided by the means and methods comprising the present invention. These include the following:

1. The present invention provides an in-situ stimulation of angiogenesis. By definition, therefore, both in-vivo and in-vitro circumstances of use and application are envisioned and expected. Moreover, the viable cells which are the location of

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PR-39 peptide and proteasome interaction, alternatively may be isolated cells; be part of living tissues comprising a variety of different cells such as endothelial cells, fibrocytes and muscle cells; and may also comprise part of specific organs in the body of a living human or animal subject. While the user shall choose the specific conditions and circumstances for practicing the present invention, the intended scope of application and the envisioned utility of the means and methods described herein apply broadly to living cells, living tissues, functional organs and systems, as well as the complete living body unit as a viable whole.

2. The present invention has a variety of different applications and uses. Of clinical and medical interest and value, the present invention provides the opportunity to stimulate angiogenesis in tissues and organs in a living subject which has suffered defects or has undergone anoxia or infarction. A common clinical instance is the myocardial infarction or chronic myocardial ischemia of heart tissue in various zones or areas of a living human subject. The present invention thus provides opportunity and means for specific site stimulation and inducement of angiogenesis under controlled conditions. The present invention also has major research value for research investigators in furthering the quality and quantity of knowledge regarding the mechanisms controlling angiogenesis under a variety of different conditions and circumstances.

3. The present invention envisions and permits a diverse range of means for introducing native PR-39 peptide or a shorter-length peptide of the oligopeptide family to a specific location, site, tissue, organ, or system in the living body. A variety of different routes of administration are available to the practitioner; and a wide and useful choice of delivery systems are conventionally available, and in accordance with good medical practice are adaptable directly for use. In this manner, not only are the means for PR-39 peptide introduction under the control of the user, but also the manner of localized application and the mode of limiting the area of peptide introduction can be chosen and controlled.

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I. Underlying Direct And Indirect Mechanisms For Initiating A  
Stimulation Of Angiogenesis

The present invention utilizes and relies upon novel and previously unknown direct and indirect mechanisms of interaction between PR-39 peptide (or its shorter-length homologs) and proteasomes in-situ as the basis for stimulation of angiogenesis in cells, living tissues, and organs. Evidence of such multiple intracellular interactions is provided by the experiments and empirical data described hereinafter. Such interactions between proteasomes (and its  $\alpha 7$  subunit in particular) and the PR-39 peptides collectively (of any size) are previously unknown; in fact, no meaningful relationship or interaction between any peptide whatsoever and intracellular proteasome function has ever been proposed or envisioned before the present invention was conceived or demonstrated empirically.

As shown experimentally hereinafter, the PR-39 peptide (and the shorter-length PR-39 derived oligopeptide family members) when introduced into the cytoplasm of viable cells will interact, directly and indirectly, with proteasomes. In some instances, the interaction between the collective of PR-39 oligopeptides and the proteasome is direct and a direct binding with the  $\alpha 7$  subunit of the proteasome occurs. In these instances, no intermediaries or cofactors are involved in the binding reaction; and such direct  $\alpha 7$  subunit binding interactions result in a selective inactivation and inhibition of proteasome function intracellularly such that expression of certain proteins such as HIF-1 $\alpha$  is increased and stimulation of angiogenesis subsequently occurs.

In addition, however, alternative mechanisms of interaction in addition to and other than direct inactivation of a proteasome subunit are deemed to exist and be in effect in-situ for members of the PR-39 peptide collective (including native PR-39 and its substituted or homologous forms). As merely an exemplary and representative instance illustrating an alternative mechanism of action, the experiments and empirical data presented hereinafter demonstrate that: the PR-39 peptide acts as a selective inhibitor of tumor necrosis factor- $\alpha$  induced I $\kappa$ B $\alpha$  degradation by proteasomes; and that PR-39 peptide inhibition of I $\kappa$ B $\alpha$  degradation is rapidly reversible (unlike the action of known inhibitory compounds such as

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5 lactacysin); and that even in the presence of PR-39 peptide, the intracellular  
expression of other proteasome-regulated proteins such as p105 and p50 NF $\kappa$ B was  
10 unchanged. These empirical findings are meaningful evidence of, harmonious  
with, and more consistent as demonstrating indirect mechanisms of interaction  
5 (rather than any direct action effect). Accordingly, the selective and reversible  
nature of PR-39 peptide activity with regard to inhibiting I $\kappa$ B $\alpha$  degradation is  
15 perceived to be due to a prevention of recognition of ubiquitinated I $\kappa$ B $\alpha$  by the  
proteasome and/or by a physical blockage of its entry into the interior of the  
proteasome in-situ. These indirect mechanisms of action are functional alternatives  
10 available on-demand and effective concurrently in addition to any direct action  
20 inactivation mechanisms.

To obtain a demonstrable proteasome interaction and effect in-situ, the  
introduction of native PR-39 peptide (or one of its substituted forms or its shorter-  
25 length homologs) is a necessary prerequisite; and the presence of sufficient PR-39  
15 peptide (or its substituted or homologous equivalent) quantitatively to interact  
selectively with proteasomes intracellularly within viable cells can be achieved  
under both in-vivo conditions and in-vitro experimental circumstances.

30 The methodology and means provided by the present invention for  
selectively inhibiting proteolysis and stimulating angiogenesis within viable cells is  
20 therefore directed at and focused upon the intracellular degradation capability and  
functional activity of proteasomes. Such selective inhibition and/or disruption of  
35 proteasome-mediated degradation is achieved via the introduction of native PR-39  
peptide or a member of the shorter-length PR-39 derived oligopeptide family in a  
therapeutic regimen of treatment.

## 25 II. Proteasomes

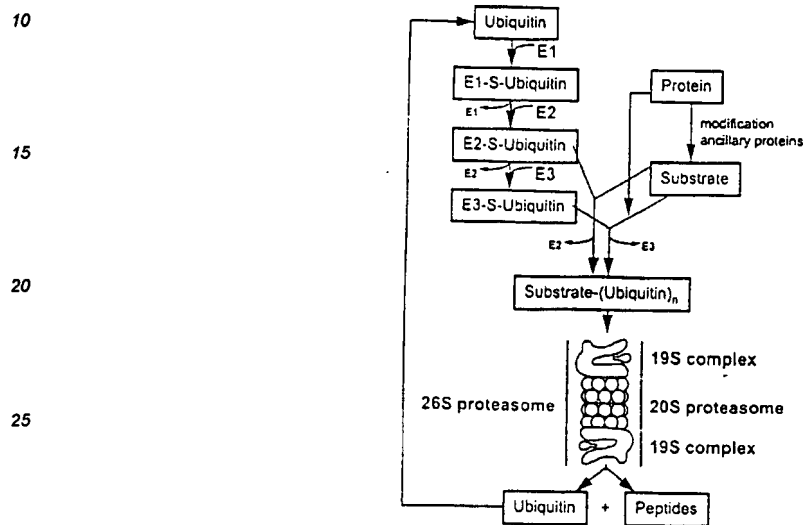
45 The proteasome is a component of the ubiquitin-proteasome-dependent  
proteolysis system. This system plays a major role in the turnover of intracellular  
30 proteins, of misfolded proteins, and in the selective degradation of key proteins.  
Controlled protein degradation is an important and efficient way to remove  
50 nonfunctional proteins and/or to regulate the activity of key proteins. Target

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5 proteins are selectively recognized by the ubiquitin system and subsequently  
marked by covalent linkage of multiple molecules of ubiquitin, a small conserved  
10 protein. The polyubiquitinated proteins are degraded by 26S proteasome. This  
complex, however, is composed of two large subcomplexes: the 20S proteasome  
5 constituting the proteolytic core and the 19S regulatory complex which confers  
polyubiquitin binding and energy dependence. A simplified scheme of the  
15 ubiquitin pathway is depicted by Flow Scheme A below.

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## Flow Scheme A



\* Schematic representation of the proteasome-ubiquitin pathway. Ubiquitin is first activated by a ubiquitin-activating enzyme (UBA or E1) and passed on to a ubiquitin-conjugating protein (UBC or E2). Ubiquitin is then linked directly, or with the help of ubiquitin ligases (E3), via an isopeptide bond to a lysine residue of the substrate protein. Polyubiquitinated proteins are recognized and selectively degraded by the 26S proteasome, yielding reusable ubiquitin molecules and peptides of 3 to 15 amino acids. Conversion of a protein into a substrate for ubiquitination can in certain cases occur after posttranslational modification or association with ancillary factors. Proteins can also be recognized by an E3 ubiquitin ligase without prior modification or association.

\* Reproduced from Gerards *et al.*, *CMLS* 54: 253-262 (1998)

5 A substantial quantum of research has been conducted to understand the  
architecture, assembly, and molecular biology of the proteasome. Merely  
representative of scientific publications in this field are the following, the  
10 individual texts of which are expressly incorporated by reference herein: Goldberg  
5 et al., Biol. Chem. 378: 131-140 (1997); Tanaka, K., Biochem. Biophys. Res.  
Commun. 247: 537-541 (1998); Baumeister et al., Cell 92: 367-380 (1998);  
15 Gerards et al., CMLS 54: 253-262 (1998); Maurizi, M.R., Curr. Biol. 8: R453-  
R456 (1998); Rechsteiner et al., J. Biol. Chem. 268: 6065-6068 (1993); Gerards  
et al., J. Mol. Biol. 275: 113-121 (1998); Fenteany, G. and S. Schreiber, J. Biol.  
10 Chem. 273: 8545-8548 (1998); and Oikawa et al., Biochem. Biophys. Res.  
Commun. 246: 243-248 (1998).

#### The 20S proteasome

25 The degrading component in ubiquitin-dependent proteolysis is the 26S  
15 proteasome. The catalytic core of this complex is the 20S proteasome, which is  
highly conserved and can be found in eukaryotes, archaeobacteria, and some  
eubacteria. In eukaryotes, the amount of proteasomes can constitute up to 1 % of  
30 the cell content, depending on the average protein breakdown rates of the organ.  
Proteasomes are localized in the nucleus and the cytosol, sometimes colocalizing or  
20 associating with the cytoskeleton. [See for example: Hilt, W. and D.H. Wolf,  
35 Trends Biochem. Sci. 21: 96-102 (1996); Ciechanover, A., Cell 79: 13-21 (1994);  
Jentsch, S. and S. Schlenker, Cell 82: 881-884 (1995); Coux et al., Annu. Rev.  
Biochem. 65: 807-847 (1996); Dahlmann et al., FEBS Lett. 251: 125-131 (1989);  
40 Tamura et al., Curr. Biol. 5: 766-774 (1995); Machiels et al., Eur. J. Cell Biol.  
25 66: 282-292 (1995); Scherrer, K. and F. Bey, Prog. Nucleic Acid Res. Mol. Biol.  
49: 1-64 (1994); and Gerards et al., CMLS 54: 253-262 (1998)].

45 The first description of a "cylinder-shaped" complex with proteasome-like  
features dates back to the late 1960s. The plethora of names given to it  
subsequently is a reflection of the problems that were encountered over a period of  
30 two decades in trying to define its biochemical properties and cellular functions.  
Enzymological studies revealed an array of distinct proteolytic activities and led to  
50 a consensus name, 'multicatalytic proteinase'. This name, however, was soon

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replaced by a new one, the 'proteasome' emphasizing its character as a molecular machine.

At about the same time, it was found that the occurrence of proteasomes was not restricted to eukaryotic cells. A compositionally simpler, but structurally strikingly similar proteolytic complex was found in the archaeon *Thermoplasma acidophilum*, which later took a pivotal role in elucidating the structure and enzymatic mechanism of the proteasome.

#### Nomenclature

The 20S proteasome was independently discovered by groups working in different fields, and hence was given a variety of different names. In 1970, Scherrer and colleagues observed ring-shaped particles in ribosome-free messenger RNA (mRNA) preparations [Sporh *et al.*, Eur. J. Biochem. **17**: 296-318 (1970)]. Subsequently, in 1979, DeMartino and Goldberg isolated a 700-kDa 'neutral protease' from rat liver [DeMartino, G.N. and A.L. Goldberg, J. Biol. Chem. **254**: 3712-3715 (1979)]. Then, in 1980 Wilk and Orlowski isolated a large protease complex from the pituitary that possessed three different catalytic activities. They called it multicatalytic protease [Wilk, S. and M. Orlowski, J. Neurochem. **35**: 1172-1182 (1980); Wilk, S. and M. Orlowski, J. Neurochem. **40**: 842-849 (1983)]. Later, Monaco and McDevitt immunoprecipitated complexes consisting of low molecular weight proteins (LMPs) with a possible role in antigen presentation [Monaco, J.J. and H.O. McDevitt, Nature **309**: 797-799 (1984)]. Also, in 1984 this particle was called prosome, referring to its presumed role in programming mRNA translation [Schmid *et al.*, EMBO J. **3**: 29-34 (1984)]. Altogether, this complex has been given 21 different names in the literature. Since all particles were shown to be identical the name 'proteasome' (which is now generally accepted) was proposed first, referring to its proteolytic and particulate nature [Arrigo *et al.*, Nature **331**: 192-194 (1988); Faulkenburg *et al.*, Nature **331**: 190-192 (1988); Brown *et al.*, Nature **353**: 355-357 (1991)].



#### Overall characteristics and properties

The 20S proteasome is the major cytosolic protease in eukaryotic cells and is the proteolytic component of the ubiquitin-dependent degradative pathway. Proteasomes are also found in some, but not all, archaeobacteria and eubacteria, and in eukaryotes. True proteasomes are composed of 28 subunits, 14 each of two different classes - non-catalytic alpha ( $\alpha$ ) and catalytically-active beta ( $\beta$ ) subunits. The subunits are arranged in rings of seven subunits, all of a single type. The 20S proteasome is a stack of four rings, two inner beta rings flanked by the alpha rings. The junction between the beta rings produces a remarkable structural feature of proteasomes - an interior aqueous cavity large enough to accommodate about 70 kDa of protein and accessible only through narrow axial channels in the rings. The catalytic sites are located on the beta subunits within the aqueous cavity. Isolation of the catalytic sites in this way, and the limited access via narrow channels, serves to compartmentalize proteolysis, allowing degradation of only those proteins that can be actively translocated into the interior of the proteasome.

#### Structure and subunit components

The 20S proteasome has a cylindrical or barrel-like structure, typically 14.8 nm in length and 11.3 nm in diameter. It is composed of 28 subunits and arranged in four stacked rings, resulting in a molecular mass of about 700 kDa. This overall structural architecture is conserved from bacteria to man.

In eukaryotes, including humans, 14 different subunits, ranging from 21 kDa to 32 kDa, are present in the complex. Based on the sequence homology with the *T. acidophilum*  $\alpha$ - or  $\beta$ -subunit, the eukaryotic subunits are divided into  $\alpha$ -type and  $\beta$ -type, respectively [Zwicki *et al.*, *Biochemistry* 31: 964-972 (1992); Heinemeyer *et al.*, *Biochemistry* 33: 12229-12237 (1994); Coux *et al.*, *Mol. Gen. Genet.* 245: 769-780 (1994)]. Table 2 shows some characteristics and alternative names of the subunits of the human and yeast 20S proteasome using the older and the new nomenclature proposed by Groll and coworkers [Groll *et al.*, *Nature* 386: 463-471 (1997)]. Immuno-electron microscopy (EM) studies also revealed that the eukaryotic  $\alpha$ -type subunits reside in the outer rings and the  $\beta$ -type subunits in the

- 14 -

inner rings. Furthermore, these studies indicated that in the eukaryotic 20S proteasome seven different subunit constitute a ring, each subunit located at a defined position [Kopp *et al.*, *L. Mol. Biol.* 229: 14-19 (1993); Kopp *et al.*, *L. Mol. Biol.* 248: 264-272 (1995); Schauer *et al.*, *L. Struct. Biol.* 111: 135-147 (1993); Kopp *et al.*, *Proc Natl Acad Sci USA* 94: 2939-2944 (1997)]. Therefore, the eukaryotic proteasome assembles as an  $\alpha_{1-7}\beta_{1-7}\beta_{1-7}\alpha_{1-7}$  particle. The typical human structure and assembly is illustrated by Table 3.

Table 2: Nomenclature and molecular masses of proteasomal subunits

	Systematic name	Human gene	Yeast gene	Molecular mass of human subunit (kDa)
5				
10	$\alpha$ 1	HsPROS27 Hslota	C7 PRS2	27.4
	$\alpha$ 2	HsC3	Y7	25.9
	$\alpha$ 3	HsC9	Y13	29.5
15	$\alpha$ 4	XAPC7 HsC6	PRE6	27.9
	$\alpha$ 5	HsZeta	PUP2	26.4
	$\alpha$ 6	HsPROS30 HsC2	PRE5	30.2
	$\alpha$ 7	HsC8	C1 PRS1	28.4
	$\beta$ 1	HsDelta Y	PRE3	25.3 (21.9)
20	$\beta$ 1i	LMP2		23.2 (20.9)
	$\beta$ 2	Z	PUP1	30.0 (24.5)
	$\beta$ 2i	MECL1		28.9 (23.8)
	$\beta$ 3	HsC10-11	PUP3	22.9
	$\beta$ 4	HsC7-1	PRE1 C11	22.8
	$\beta$ 5	MB1 X	PRE2	nd (22.4)
25	$\beta$ 5i	LMP7		30.4 (21.2)
	$\beta$ 6	HsC5	C5 PRS3	26.5 (23.3)
	$\beta$ 7	HsBPROS26 HsN3	Pre4	29.2 (24.4)

---

\* Reproduced from Gerards *et al.*, *CMLS* 54: 253-262 (1998)

Table 3: Schematic representation of the human 20S proteasome\*



\* Reproduced from Gerards *et al.*, *CMLS* 54: 253-262 (1998)

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Proteolytic activity

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The first report on the multicatalytic properties of the proteasome stems from 1983, when three different proteolytic activities were distinguished: 'trypsin-like', 'chymotrypsin-like' and 'peptidylglutamyl-peptide hydrolase' activity [Wilk, S. and M. Orlowski, L. Neurochem. 40: 842-849 (1983)]. These three proteasomal activities refer to peptide bond cleavage at the carboxyl side of basic, hydrophobic and acidic amino acid residues, respectively. They were identified using short synthetic peptide substrates and are believed to be catalyzed at independent sites - in part because the different proteolytic activities respond differentially to various activators and inhibitors. With similar approaches, at least two additional proteolytic activities have been recently described [Orlowski et al., Biochemistry 32: 1563-1572 (1993); Orlowski, M., Biochemistry 29: 10289-10297 (1990); Rivett, A.J., Biochem. J. 291: 1-10 (1993)].

The Progressive Degradation Of Protein Substrates

Recent studies have also revealed a fundamental new property of the proteasome that clearly distinguishes it from conventional proteases: i.e., this particle degrades a protein substrate all the way to small peptides, before attacking another protein substrate [Akopian et al., L. Biol. Chem. 272: 1791-1798 (1997)]. Because the proteasome's multiple active sites are located in its central chamber and because diffusion of a peptide substrate into this compartment must be a slow process, these particles function in a highly processive fashion; i.e., they have mechanisms of action to bind tightly protein substrates and to make multiple cleavages in the polypeptide before releasing the peptide products. Moreover, the ratio of new peptides generated to the number of substrate molecules consumed is constant during the reaction. In other words, as peptides accumulated, they were not hydrolyzed further, even during prolonged incubations, where up to half of the substrate molecules were consumed. Equally important, the disappearance of these substrate molecules coincided exactly with the appearance of small peptide products [Goldberg et al., Biol. Chem. 378: 131-140 (1997)]. These observations, together with the finding that the pattern of the products is independent of time, established

that processive degradation is a general feature of the 20S proteasome [Gerard et al., CMLS 54: 253-262 (1998)].

The contribution of each individual active center and proteolytic activity to the degradation of longer peptides and complete proteins is presently unknown.

Nevertheless, proteasomes are able to cleave behind most amino acids in a protein. Thus, the 20S proteasome is in fact a nonspecific endopeptidase. In addition, however, the generated (degraded) peptides fall into a rather narrow size range of 6 to 10 amino acids in length, demonstrating the existence of a kind of 'molecular ruler'. The average length of the degradation products is typically 7 to 8 amino acids; this finding is in agreement with the distance between the active sites in the proteasome. Similar nonspecific endopeptidase activity and size distribution of degradation products from whole proteins was observed for proteasomes generally and by proteasomes of human origin in particular.

Other features of the 20S proteasome degradation are also unique. While unfolded peptides are usually digested, most native proteins are resistant to proteolytic degradation by the 20S proteasome in vitro. However, denaturation of the substrate protein by oxidation or reduction of disulphide bridges can render it accessible to degradation by proteasomes. Also, small gold particles with a diameter of 2 nm containing unfolded substrate cannot enter the proteasome.

These characteristics show that a relatively narrow opening controls access to the inner proteolytic compartment of the proteasome.

### III. The PR-39 Oligopeptide Collective

Native PR-39 peptide is a substance belonging to the cathelin family of proteins; the mature peptide is 39 amino acids in length in the naturally occurring state; and the peptide is able to exert a variety of activities and cause different cellular outcomes. Although first identified as a membrane permeating antibacterial peptide found in the intestine of pigs [Agerberth et al., Eur. J. Biochem. 202: 849-854 (1991)], this peptide was subsequently isolated from wounds where it could simultaneously reduce infection and influence the action of growth factors, matrix components, and other cellular effectors involved in wound

5 repair [Gallo *et al.*, *Proc. Natl. Acad. Sci. USA* **91**: 11035-11039 (1994); Gallo  
*et al.*, *J. Invest. Dermatol.* **104**: 555 (1995)]. The structure and membrane  
10 interactions of native PR-39 peptide have also been elucidated [Cariaux *et al.*, *Eur.*  
*J. Biochem.* **224**: 1019-1027 (1994)] and the complete amino acid sequences of  
5 native PR-39 peptide and its various substituted forms have been reported [PCT  
Publication No. WO 92/22578 published 23 December 1992].

15 More recently, the native PR-39 peptide was shown to possess a syndecan-  
inducing activity in furtherance of its wound healing capabilities; and while  
renamed a "synducin", was shown to induce cellular production of two specific  
20 proteoglycans, syndecan-1 and syndecan-4, within living mesenchymal cells [U.S.  
Patent No. 5,654,273]. Overall, native PR-39 peptide has been shown to play a  
role in several inflammatory events including wound healing and myocardial  
infarction [Gallo *et al.*, *Proc. Natl. Acad. Sci. USA* **91**: 11035-11039 (1994);  
25 Li *et al.*, *Circ. Res.* **81**: 785-796 (1997)]; and the native peptide has been shown to  
15 be taken up rapidly by a number of different cell types including mesenchymal cells  
and endothelial cells [Chan, Y.R. and R.L. Gallo, *J. Biol. Chem.* **273**: 28978-  
28985 (1998)].

#### The PR-39 peptide grouping

20 Native PR-39 peptide is composed of the 39 amino acid sequence shown  
below (and also by Table 4).

35  
40  
25 PR-39: Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro-  
Pro-Phe-Phe-Pro-Pro-Arg-Leu-Pro-Pro-Arg-Ile-Pro-Pro-Gly-Phe-  
Pro-Pro-Arg-Phe-Pro-Pro-Arg-Phe-Pro

45 As conventionally known and reported [see for example, U.S. Patent No.  
5,654,273], the specific peptide can be substituted using conservative substitutions  
of amino acids having the same or functionally equivalent charge and structure,  
30 except for the required amino acid sequence "Arg-Arg-Arg" at the N-terminus and  
the intermediate amino acid sequences "Pro-Pro-X-X-Pro-Pro-X-X-Pro" and "Pro-  
50 Pro-X-X-X-Pro-Pro-X-X-Pro" where X can be substituted freely using any amino

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acid. Thus, all of the preferred substituted amino acid sequences are of about the same size and each differ from the native PR-39 peptide sequence only by substitutions in the intermediate portions of the structure.

The PR-39 derived oligopeptide family

In addition to the conventionally known native PR-39 peptide amino acid residue sequence and its readily recognizable substituted forms as described above, an entirely novel and unforeseen family of PR-39 derived oligopeptide structures is provided by the present invention for use. This previously unknown family of PR-39 derived oligopeptides is constituted of members which individually will cause a selective inhibition of proteasome-mediated degradation of peptides in-situ after introduction intracellularly to a viable cell.

Each member of this PR-39 derived oligopeptide family presents characteristics and properties which are commonly shared among the entire membership. These include the following:

- (i) each peptide sequence is less than 39 amino acid residues in length in every embodiment, and preferably is less than 20 residues in size in the best mode;
- (ii) each short-length peptide sequence is at least partially homologous (or analogous) with the N-terminal amino acid residues of the native PR-39 peptide, and preferably is completely identical or markedly similar to the N-terminal end residues of the native PR-39 peptide;
- (iii) each short-length peptide is able to interact in-situ with at least the  $\alpha 7$  subunit of such proteasomes as are present within the cytoplasm of the cell; and
- (iv) each short-length peptide sequence is able to alter markedly the proteolytic activity of proteasomes with an interacting  $\alpha 7$  subunit such that a selective increased expression of specific proteins (such as I $\kappa$ B $\alpha$  and HIF-1 $\alpha$ ) occurs in-situ.

Merely as illustrative examples and preferred embodiments of the broad membership constituting this PR-39 derived oligopeptide family, the members comprising 15, 11 and 8 amino acid residues respectively in length are presented



- 21 -

below as the PR15, PR11, and PR8 entities respectively. For comparison purposes only, the complete amino acid sequence of the native PR-39 peptide is presented as well.

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15  
20  
25  
30

PR-39: 1 2 3 4 5 6 7 8 9 10 11 12 13  
Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-  
14 15 16 17 18 19 20 21 22 23 24 25 26  
Pro-Pro-Pro-Phe-Phe-Pro-Pro-Arg-Leu-Pro-Pro-Arg-Ile-  
27 28 29 30 31 32 33 34 35 36 37 38 39  
Pro-Pro-Gly-Phe-Pro-Pro-Arg-Phe-Pro-Pro-Arg-Phe-Pro

PR-15: 1 2 3 4 5 6 7 8 9 10 11 12 13  
Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-  
14 15  
Pro-Pro

PR-11: 1 2 3 4 5 6 7 8 9 10 11  
Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg

PR-8: 1 2 3 4 5 6 7 8  
Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr

#### The PR-39 Oligopeptide Collective

Terminology and nomenclature often pose problems for the reader as to what precisely is meant. Accordingly, for definitional purposes, avoidance of ambiguities, and clarity of understanding, the following terms and titles will be employed herein. The term "PR-39 peptides grouping" includes by definition the native PR-39 structure and all substituted forms conventionally known of the naturally occurring 39 length amino acid sequence. In distinction, the term "PR-39 derived oligopeptide family" and its members includes by definition all the previously unknown shorter-length homologs and analogs of the native PR-39 structure as described above. Finally, the umbrella term and category title "PR-39 oligopeptide collective" includes by definition both the 'PR-39 peptide grouping' as well as the 'PR-39 derived oligopeptide family' members, and identifies any and all individual structures falling into either of the two subset categories.

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Table 4:

(1) GENERAL INFORMATION:  
 (i) APPLICANT: Children's Medical Center Corporation  
 (ii) TITLE OF INVENTION: Synducin Mediated Modulation of  
 Tissue Repair  
 (iii) NUMBER OF SEQUENCES: 4  
 (iv) CORRESPONDENCE ADDRESS:  
 (A) ADDRESSEE: Patrea L. Pabst  
 (B) STREET: 2800 One Atlantic Center  
 1201 West Peachtree  
 (C) CITY: Atlanta  
 (D) STATE: Georgia  
 (E) COUNTRY: USA  
 (F) ZIP: 30109-3450  
 (v) COMPUTER READABLE FORM:  
 (A) MEDIUM TYPE: Floppy disk  
 (B) COMPUTER: IBM PC compatible  
 (C) OPERATING SYSTEM: PC-DOS/MS-DOS  
 (D) SOFTWARE: PatentIn Release #1.0, Version #1.25  
 (ix) TELECOMMUNICATION INFORMATION:  
 (A) TELEPHONE: (404)-873-8794  
 (B) TELEFAX: (404)-815-8795  
 (2) INFORMATION FOR SEQ ID NO:1:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 39 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear  
 (ii) MOLECULE TYPE: peptide  
 (iii) HYPOTHETICAL: NO  
 (iv) ANTI-SENSE: NO  
 (x) PUBLICATION INFORMATION:  
 (A) AUTHORS: Lee, Jong-Young  
 Boman, Hans G.  
 Mutt, Viktor  
 Jornvall, Hans  
 (B) TITLE: Novel Polypeptides And Their Use  
 (C) JOURNAL: PCT WO 92/22578  
 (G) DATE: 12/23/92  
 (K) RELEVANT RESIDUES IN SEQ ID NO:1: FROM 1 TO 39  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:  
 Arg Arg Arg Pro Arg Pro Pro Tyr Leu Pro Arg Pro Arg Pro  
 Pro Pro 1 5 10  
 15  
 Phe Phe Pro Pro Arg Leu Pro Pro Arg Ile Pro Pro Gly Phe  
 Pro Pro 20 25 30  
 40  
 Arg Phe Pro Pro Arg Phe Pro  
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Synthesis

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The PR-39 peptide can be synthesized using standard amino acid synthetic techniques. An example is the conventionally used solid phase synthesis [Merrifield, J., L. Am. Chem. Soc. 85: 2149 (1964)] described in U.S. Patent No. 4,244,946, wherein a protected alpha-amino acid is coupled to a suitable resin, to initiate synthesis of a peptide starting from the C-terminus of the peptide. Other methods of peptide synthesis are described in U.S. Patent Nos. 4,305,872 and 4,316,891, the teachings of which are incorporated herein. These methods can be used to synthesize peptides having identity with the native PR-39 peptide amino acid sequence described herein, or to construct desired substitutions or additions of specific amino acids, which can be screened for content and evaluated for activity. PR-39 can also be commercially obtained from Magainin, Inc. (Plymouth Meeting, PA).

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15 Pharmaceutical Formats

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After synthesis or purchase, the PR-39 peptides (as a family of homologs and analogs with substituted amino acid residues) can be introduced as a peptide-containing preparation in a pharmaceutically acceptable format.

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The PR-39 can be administered and introduced in-vivo systemically, topically, or locally. The peptide can be administered as the peptide or as a pharmaceutically acceptable acid- or base-addition salt, formed by reaction with an inorganic acid (such as hydrochloric acid, hydrobromic acid, perchloric acid, nitric acid, thiocyanic acid, sulfuric acid, and phosphoric acid); or with an organic acid (such as formic acid, acetic acid, propionic acid, glycolic acid, lactic acid, pyruvic acid, oxalic acid, malonic acid, succinic acid, maleic acid, and fumaric acid); or by reaction with an inorganic base (such as sodium hydroxide, ammonium hydroxide, potassium hydroxide); or with an organic base (such as mono-, di-, trialkyl and aryl amines and substituted ethanolamines).

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PR-39 peptide and any of the PR-39 derived oligopeptide family members may also be conjugated to sugars, lipids, other polypeptides, nucleic acids and

5 PNA; and function in-situ as a conjugate or be released locally after reaching a  
targeted tissue or organ. The PR-39 family of peptides may also be linked to  
10 targeting compounds for attachment in-situ to a specific cell type, tissue or organ.

5 IV. Means For Introduction Of PR-39 Peptide  
And/Or Its Shorter-Length Derived Homologs

15 DNA Fragments and Expression Vectors

A variety of means and methods are conventionally known and presently  
10 available to the user or practitioner of the present invention in order to introduce  
PR-39 peptide (or a derived oligopeptide family member) to living cells and  
20 tissues. One desirable means uses a prepared DNA sequence fragment encoding  
the PR-39 peptide (or a shorter-length homolog) in a suitable vector as the means  
25 of introduction to the intended target in-situ. These means for delivery envision  
and include in-vivo use circumstances; ex-vivo specimens and conditions; and in-  
15 vitro cultures. In addition, the present invention intends and expects that the  
prepared DNA sequence fragment coding for PR-39 peptide (or shorter-length  
30 homologs) has been inserted in a suitable expression vector and will be used in a  
route of administration for delivery to living tissues comprising endothelial cells,  
20 and typically vascular endothelial cells which constitute the basal layer of cells  
within capillaries and blood vessels generally. Clearly, the cell recipients  
35 themselves are thus eukaryotic in origin, typically mammalian cells from human  
and animal sources; and most typically would include the higher orders of  
mammals such as humans and domesticated mammalian animals kept as pets or  
40 sources of food intended for future consumption. Accordingly, the range of  
animals includes all domesticated varieties involved in nutrition including cattle,  
sheep, pigs and the like; as well as those animals typically used as pets or raised  
45 for commercial purposes including horses, dogs, cats, and other living mammals  
typically living with and around humans.

30 Clearly, the expression vectors must be suitable for transfection of  
endothelial cells in living tissues of mammalian origin and thus be compatible with  
50 that type and condition of cells under both in-vivo and/or in-vitro conditions. The

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expression vectors thus typically include plasmids and viruses as expression vectors.

Also, both the plasmid based vectors and the viral expression vectors constitute conventionally known means and methods of introduction which are conventionally recognized today as "gene therapy" modes of delivery. However, this overall approach is not the only means and method of delivery available for the present invention.

Direct Introduction of Previously Synthesized PR-39 Peptides or a PR-39 Derived Oligopeptide Family Member

PR-39 peptide or an oligopeptide family member can be introduced directly as a synthesized compound to living cells and tissues via a range of different delivery means. These include the following.

1. Intracoronary delivery is accomplished using catheter-based deliveries of synthesized PR-39 peptide (or homolog member) suspended in a suitable buffer (such as saline) which can be injected locally (i.e., by injecting into the myocardium through the vessel wall) in the coronary artery using a suitable local delivery catheter such as a 10mm InfusaSleeve catheter (Local Med, Palo Alto, CA) loaded over a 3.0mm x 20mm angioplasty balloon, delivered over a 0.014 inch angioplasty guidewire. Delivery is typically accomplished by first inflating the angioplasty balloon to 30 psi, and then delivering the protein through the local delivery catheter at 80 psi over 30 seconds (this can be modified to suit the delivery catheter).
2. Intracoronary bolus infusion of PR-39 peptide (or a short-length homolog) synthesized previously can be accomplished by a manual injection of the substance through an Ultrafuse-X dual lumen catheter (SciMed, Minneapolis, MN) or another suitable device into proximal orifices of coronary arteries over 10 minutes.
3. Pericardial delivery of synthesized PR-39 peptide (or a shorter-length homolog) is typically accomplished by instillation of the peptide-containing solution into the pericardial sac. The pericardium is accessed via a right atrial puncture, transthoracic puncture or via a direct surgical approach. Once the access is established, the peptide material is infused into the pericardial cavity and the

- 26 -

5 catheter is withdrawn. Alternatively, the delivery is accomplished via the aid of  
slow-release polymers such as heparin-alginate or ethylene vinyl acetate (EVAc).  
10 In both cases, once the PR-39 peptide (or homolog) is integrated into the polymer,  
the desired amount of PR-39/polymer is inserted under the epicardial fat or secured  
5 to the myocardial surface using, for example, sutures. In addition, the PR-39/  
polymer can be positioned along the adventitial surface of coronary vessels.

15 4. Intramyocardial delivery of synthesized PR-39 peptide (or a shorter-length  
homolog) can be accomplished either under direct vision following thoracotomy or  
using thoracoscope or via a catheter. In either case, the peptide containing solution  
20 is injected using a syringe or other suitable device directly into the myocardium.

Up to 2 cc of volume can be injected into any given spot and multiple locations (up  
25 to 30 injections) can be done in each patient. Catheter-based injections are carried  
out under fluoroscopic, ultrasound or Biosense NOGA guidance. In all cases after  
catheter introduction into the left ventricle the desired area of the myocardium is  
15 injected using a catheter that allows for controlled local delivery of the material.

#### 30 Pharmaceutical Carriers Of PR-39 Peptides or a PR-39 Derived Oligopeptide Family Member

A range of suitable pharmaceutical carriers and vehicles are known  
20 conventionally to those skilled in the art. Thus, for parenteral administration, the  
compound will typically be dissolved or suspended in sterile water or saline.

35 For enteral administration, the PR-39 peptide or homologous oligopeptide of  
choice will be typically incorporated into an inert carrier in tablet, liquid, or  
capsular form. Some suitable carriers are starches and sugars; and often include  
40 25 lubricants, flavorings, binders, and other materials desirable in tablet making  
procedures.

The PR-39 peptide and oligopeptide family of compounds can also be  
45 administered topically by application of a solution, cream, gel, or polymeric  
material (for example, a Pluronic™, BASF).

30 As an alternative, the chosen peptide can be administered in liposomes or  
microspheres (or microparticles), which can be injected for local or systemic  
50 delivery. Methods for preparing liposomes and microspheres for administration to

- 27 -

5 a patient are conventionally known to those skilled in the art. For example, U.S.  
Patent No. 4,789,734 describes methods for encapsulating biological materials in  
liposomes. Essentially, the material is dissolved in an aqueous solution, the  
10 appropriate phospholipids and lipids added, along with surfactants if required, and  
5 the material dialyzed or sonicated, as necessary. See also, G. Gregoriadis,  
Chapter 14, "Liposomes", Drug Carriers in Biology and Medicine, chap. 14, pp.  
287-341 (1979). Microspheres formed of polymers or proteins are well known to  
15 those skilled in the art, and can be tailored for passage through the gastrointestinal  
tract directly into the bloodstream. Alternatively, the compound can be  
10 incorporated and the microspheres, or composite of microspheres, implanted from  
20 days to months. See, for example, U.S. Patent Nos. 4,906,474; 4,925,673; and  
3,625,214.

#### 25 Exemplary Introductions And Preferred Routes of Administration

15 A variety of approaches, routes of administration, and delivery methods  
have been identified herein and are available for introduction of PR-39 peptide and  
the derived family of oligopeptides. It is envisioned, however, that a majority of  
30 the approaches and routes of administration described herein will be medical  
applications and specific clinical approaches intended for use with individual human  
20 patients having specified medical problems and diagnosed pathologies. It is  
35 expected, accordingly, that the reader is familiar generally with the typical clinical  
human problem, pathology, and medical conditions described herein; and therefore  
will be able to follow and easily understand the nature of the intervention clinically  
40 using the present invention and the intended outcome and result of the clinical  
25 treatment - particularly as pertains to the stimulation of angiogenesis under in-vivo  
treatment conditions. A representative listing of preferred clinical approaches is  
given by Table 5 below.

Table 5

## Preferred Routes of Administration

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Catheter-based (intracoronary) injections and infusions;

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Direct myocardial injection

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(intramyocardial guided);

Direct myocardial injection

10

(direct vision-epicardial-open chest or under thoroscope guidance);

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Local intravascular delivery;

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Liposome-based delivery;

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Delivery in association with receptor-specific peptides;

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Oral delivery;

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In instances of peripheral vascular disease:

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- intramuscular injection

- intraarterial injection and/or infusion.

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## V. Experiments and Empirical Data

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To demonstrate the merits and value of the present invention, a series of planned experiments and empirical data are presented below. It will be expressly understood, however, that the experiments described and the results provided are merely the best evidence of the subject matter as a whole which is the invention; and that the empirical data, while limited in content, is only illustrative of the scope of the invention envisioned and claimed.

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10 Introduction

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Proteolytic degradation in mammalian cells is known to proceed via two distinct pathways: lysosome-dependent degradation and proteasome-dependent. The proteasome in its pathway plays a key role in proteolysis of intracellular proteins which are marked for degradation by the ubiquitin system. The multienzyme complex involved in these events, the 26S proteasome, consists of a 20S catalytic proteasome "core" and two 19S caps that bind ubiquitylated proteins, as has been described in detail previously herein. Proteasome-mediated proteolysis is a principal event quantitatively controlling intracellular levels of a number of different proteins including hypoxia-inducing factor (HIF)-1 $\alpha$ , heat shock protein HSP70, protooncogenes c-Fos, c-Jun and c-Mos, NF $\kappa$ B inhibitor I $\kappa$ B $\alpha$ , and various cyclins. In addition, the proteasome also is known to play a critical role in the specific processing and presentation of major histocompatibility complex (MHC) class I-restricted antigens as well as provide partial proteolytic cleavage of p105 NF $\kappa$ B to the active p50 subunit.

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The PR-39 peptide, belonging to the cathelin family of proteins, plays an important role in several inflammatory events including wound healing and myocardial infarction. The PR-39 peptide typically is rapidly taken up by a number of different cell types including endothelial cells; and prolonged treatment with PR-39 peptide leads to increased cell growth and angiogenesis. However, the mechanism of action for this peptide activity has yet to be understood or defined. The experiments and data presented below reveal for the first time the nature and detailed intracellular actions exerted by the PR-39 peptide.

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### Methods and Materials:

#### **Yeast two-hybrid screening**

Two-hybrid screening was carried out using MATCHMAKER GAL4 System 2 (Clontech) with exon 4 of the porcine PR-39 gene as a bait to screen the mouse embryo 3T3 cDNA library in yeast CG1945.

#### **Cell culture studies**

U937 cells (ATCC) grown in RPMI medium 1640 with 10% FBS (Gibco-BRL) and ECV cells were treated with synthetic PR-39, lactacystin (CalBiochem, 426100) or MG132 (CalBiochem, 474790) at concentration indicated in the presence of 100 mM cyclohexamide 20 mM chloroquine [Merin *et al.*, *J. Biol. Chem.* **273**: 6373-6379 (1995)]. After 45 min. of incubation, TNF $\alpha$  (1 ng/ml) was added. After 5 min of 37°C incubation, the cells were lysed in SDS-PAGE loading buffer. Following SDS-PAGE of the total protein extract, I $\kappa$ B- $\alpha$  and NF- $\kappa$ B p105 p50 expressions were determined by Western blotting with anti-human antibodies (Santa Cruz, sc-203, sc114G). For studies of HIF-1 $\alpha$  and VEGF expression, ECV cells were cultured in a hypoxia chamber (5% CO<sub>2</sub>/95% N<sub>2</sub>) at 37°C for 16 hr. HIF-1 $\alpha$  was immunoprecipitated with anti-HIF-1 $\alpha$  mAb (OZ12 1:5) in RIPA buffer and Western blotting with anti-HIF-1 $\alpha$  mAb (OZ15 1:10) (courtesy of Dr. A. King, DFCI, Boston). VEGF expression was shown by Western blotting of hypoxia treated ECV cell lysate with anti-human VEGF antibody (Santa Cruz, sc-152). For HSP70 expression, U937 was treated for 3 hr, harvest with SDS-PAGE loading buffer, Western blotting with anti-human HSP70 polyclonal antibody (Santa Cruz, sc-1060).

#### **In-vitro proteasome activity assays**

Rabbit muscle 20S proteasome preparation (courtesy of Dr. M. Sherman, BBRI, Boston) was used for all studies. For determination of proteasome activity, 5  $\mu$ l of 1:10 diluted proteasome preparation was incubated at room temperature in eukaryotic proteasome assay buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA and 0.01% SDS) with 20  $\mu$ M proteasome substrates (CalBiochem, 539140-3) and

- 31 -

PR-39 or other proteasome inhibitor at indicated concentration [Rock *et al.*, *Cell* 78: 761-771 (1994)]. The extent of substrate degradation was monitored continuously by fluorescence spectrophotometry (380 nm excitation, 460nm emission Hitachi F-2000) for 10 min.

## Experiment 1:

This experiment was designed to reveal the ability of PR-39 peptide to affect proteasome function. To test this capability, the effect of PR-39 administration upon  $\alpha 7$  subunit processing was empirically determined. The results are illustrated by Figs. 1A-1D respectively.

Experimentally, a peptide corresponding to the 4th exon porcine PR-39 gene sequence was used to generate a rabbit polyclonal antibody RPE4. Full length porcine cDNA (containing leader sequence) and a sequence corresponding to the 4th exon of porcine PR-39 gene were cloned into eukaryotic expression vector pGRES-2 (USB). These expression constructs were then used to stably transfect an immortalized human endothelial cell line (ECV304, ATCC). For co-immunoprecipitation, wild type ECV, full length PR-39 (ECV-PR39) and exon 4 PR39 and exon 4 PR39 (ECV-E4) transfected cells were cultured in Medium 199 with 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cells were lysed with RIPA buffer; immunoprecipitated with 10  $\mu$ g affinity purified rabbit anti-PR39 antibody; and following Protein A-Sepharose purification and SDS-PAGE, subjected to immunoblotting with 1:1000 mouse anti-HC8 mAb (Affiniti Research Products Limited UK, PW8110).

Figs. 1A-1D show the interactions of PR-39 peptide and the  $\alpha 7$  subunit of proteasomes. Fig. 1A recites the cDNA sequence of cloned mouse  $\alpha 7$  subunit (top; GeneBank accession number AF055983) and corresponding human HC8 subunit of 20S proteasome. Fig. 1B shows the sequence alignment of C-terminal tails mouse  $\alpha$  subunits of 20S proteasome. Fig. 1C shows a deletion analysis of  $\alpha 7$ -PR39 binding. Deletion mutants of the mouse  $\alpha 7$  subunit were cloned into an yeast-two hybrid vector and the extent of growth of *lacZ*<sup>+</sup> colonies on selective medium following co-transformation with PR-39 construct in the yeast CG1945 was determined. It is noted that only full length  $\alpha 7$  construct was able to bind to

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PR-39. Finally, Fig. 1D shows the co-immunoprecipitation of PR-39 and  $\alpha 7$  subunit in ECV cells.

It will be noted also that Fig. 1 represents the evidence of four clones growing on selective media and demonstrating lacZ staining. All four clones encoded overlapping identical cDNA sequences highly homologous to the human sequence of  $\alpha 7$  (HC8) subunit of proteasome (Fig. 1A). Similar to all  $\alpha$  subunits of the 20S proteasome, the cloned mouse protein possesses a highly conserved N-terminal region; in addition it demonstrated the presence of 16 amino acid long C-terminal sequence found in some but not all  $\alpha$  subunits (Fig. 1B). Deletion analysis showed that the presence of both C-terminal as well as N-terminal amino acids sequences was required for PR-39 binding (Fig. 1C). In order to confirm the PR39- $\alpha 7$  subunit interaction in-vivo, anti-PR39 antibody was used to immunoprecipitate PR39 protein from ECV-PR39, ECV-E4 and mock-transfected ECV cells. Western blotting of the immunoprecipitate from ECV-PR39 and ECV-E4 but not wild type ECV cells with anti- $\alpha 7$  subunit antibody demonstrated the presence of a 29kDa band corresponding to the known size of  $\alpha 7$  subunit protein (Fig. 1D). The evidence therefore reveals that PR39 peptide interacts with  $\alpha 7$  subunit of proteasome in ECV cells.

#### Experiment 2:

To test the ability of PR-39 peptide to affect proteasome function in-vivo, the effect of PR-39 peptide administration on I $\kappa$ B $\alpha$  processing was assessed. The results are illustrated by Figs. 2A-2D respectively.

Fig. 2A shows a Western analysis of I $\kappa$ B $\alpha$  expression in ECV cells. The results show that pretreatment of cultured ECV cells with lactacystin (10  $\mu$ M, 4th lane) or stable expression of full length (ECV-PR39) or PR39 exon 4 (ECV-E4) constructs inhibited TNF- $\alpha$ -induced degradation of I $\kappa$ B.

Thus, tumor necrosis factor (TNF)- $\alpha$  induces rapid degradation of I $\kappa$ B $\alpha$  - a function that is blocked by the proteasome inhibitor lactacystin. However, Western analysis of I $\kappa$ B $\alpha$  levels after TNF- $\alpha$  treatment demonstrated comparable levels of I $\kappa$ B $\alpha$  expression in both ECV-PR39 and ECV-E4 cells to that seen in ECV cells pre-treated with lactacystin.

Fig. 2B shows the effect of PR39, MG132 and lactacystin pretreatment on I $\kappa$ B $\alpha$  expression in U937 cells following TNF- $\alpha$  treatment. Note similar extent of inhibition of I $\kappa$ B $\alpha$  degradation by TNF- $\alpha$  following pretreatment with PR39, MG132 or lactacystin. Thus, it is clear that pretreatment of U937 cells with PR39 blocked TNF- $\alpha$  induced I $\kappa$ B $\alpha$  degradation in a manner that was similar to the degree of inhibition seen with MG132 and lactacystin.

Fig. 2C demonstrates the reversibility of PR39 inhibition of proteasome activity. U937 cells were pretreated with PR39, MG132 or lactacystin for 45 min. After that time, the cells were extensively washed with fresh medium. 45 min later TNF- $\alpha$  (1 ng/ml) was added to the medium and the extent of I $\kappa$ B $\alpha$  degradation was determined 10 min later by Western blotting. Note preservation of I $\kappa$ B $\alpha$  in lactacystin-treated cells but not PR39-treated cells. Thus, unlike lactacystin but similar to MG132, PR-39 peptide mediated inhibition of I $\kappa$ B $\alpha$  degradation was rapidly reversible.

Finally, to show that PR-39 inhibition of I $\kappa$ B $\alpha$  degradation affected NF $\kappa$ B-dependent transcription, ECV cells were transiently transfected with a NF $\kappa$ B-Luc reporter construct containing a tandem of four NF $\kappa$ B binding sites in front of luciferase cDNA. The results of Fig. 2D show that stimulation with TNF- $\alpha$  induced a significant increase in luciferase activity that was completely inhibited by pretreatment with PR39.

Accordingly, the true functional significance of PR39-mediated inhibition of I $\kappa$ B $\alpha$  degradation in ECV cells transiently transfected with pNF $\kappa$ B-Luc reporter vector (Clontech) is clearly shown by Fig. 2D. Pre-treatment with PR39 completely inhibited TNF- $\alpha$ -induced increase in luciferase activity. \* $p < 0.01$  vs. control (Luc activity in the absence of TNF- $\alpha$ ).

#### Experiment 3:

To demonstrate directly the ability of PR-39 peptide to inhibit proteasome-mediated protein degradation, preparations of eukaryotic 20S proteasomes were tested for their ability to induce proteolysis of various synthetic peptides in-vitro. The results are graphically illustrated by Figs. 3A-3D respectively.

For determination of proteasome activity, 5  $\mu$ l of 1:10 diluted rabbit muscle 20S proteasome preparation (courtesy of Dr. M. Sherman, BBRI, Boston) was incubated at room temperature in an assay buffer (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA and 0.01% SDS) with 20  $\mu$ M of four different proteasome substrates (CalBiochem, S39140-3) and PR39 or other proteasome inhibitor at indicated concentration. The extent of substrate degradation was monitored continuously by fluorescence spectrophotometry (380 nm excitation, 460 nm emission Hitachi F-2000) for 10 min.

Figs. 3A-3D reveal that the PR-39 peptide inhibited, in a dose-dependent manner, degradation of all 4 peptides tested. PR-39 peptide was as potent as lactacystin or MG132 in inhibiting degradation in three of the four peptides tested and was considerably more potent in inhibiting degradation of the Z-Leu-Leu-Glu-AMC peptide.

#### Experiment 4:

To test the effect of PR39 treatment on cellular levels of other proteasome-dependent proteins, the in-vivo expression of p105 and p50 NF $\kappa$ B, HSP70 and HIF-1 $\alpha$  within transfected and wild type ECV cells was determined. The results are illustrated by Figs. 4A-4C respectively.

Fig. 4A shows the results of a Western blot analysis of HIF-1 $\alpha$ , p50 and p105 NF $\kappa$ B expression in wild type ECV cells and ECV-E4 and ECV-PR39 clones. It is noted that an increase in HIF-1 $\alpha$  expression occurs (but not p105 or p50 NF $\kappa$ B expression) in ECV-E4 and ECV-PR 39 clones. Thus, while there was a significant increase in expression of HIF-1 $\alpha$  and I $\kappa$ B in PR39 transfected or treated compared to wild type cells, there was no significant change in expression of either HSP70 or NF $\kappa$ B - demonstrating that effects of PR39-proteasome interaction are selective.

Since increased expression of HIF-1 $\alpha$  is known to result in increased transcription of a number of angiogenesis-related molecules including VEGF, Northern analysis of VEGF mRNA levels in wild type and PR39 transfected ECV cells was performed. The results are shown by Fig. 4B. As expected, there was a

- 35 -

significant increase in expression of both of these genes in ECV-PR39 and ECV-E4 cells compared to ECV controls.

Finally, exposure to proteasome inhibitor lactacystin is known to induce rapid cell death. To test the effect of PR39 on cell survival, growth rates of ECV cells treated with PR39 peptide were assessed. The results are shown by Fig. 4C. 50,000 ECV cells were cultured in 10% FBS-M199 in the absence (control) or presence of 10  $\mu$ M of PR39, lactacystin or MG132. Note that while exposure to PR39 did not affect cell growth, exposure to lactacystin or MG132 substantially inhibited cell growth. Thus, following 3 days of PR39 exposure, treated cells demonstrated normal growth compared to controls while those cells exposed to lactacystin demonstrated markedly reduced survival.

#### Experiment 5:

To demonstrate the stimulation of angiogenesis directly in living cells and tissues via the introduction of PR-39 peptide, a mice matrigel assay system was employed. Growth factor-depleted Matrigel pellets containing 5  $\mu$ g of PR39, 50 ng of FGF2 or saline (control) were inserted intraperitoneally into C57BL/6 mice. Ten days later the pellets were removed, sectioned and stained with anti-CD31 antibody. The number of vessels was determined in multiple sections using a digital camera and Optimas 5.0 software. The results are shown by Figs. 5A-5C and Fig. 6 respectively.

Figs. 5A-5C are representative sections from control, PR39 and FGF2 impregnated Matrigel pellets and Fig. 6 provides a quantitative analysis of vascularity. Clearly, the results of the representative sections and the graphic quantitative evaluations demonstrate that insertion of growth-factor depleted Matrigel pellet containing PR-39 peptide induced intense vessel growth that exceeded that seen with implantation of pellets containing 50 ng/ml of bFGF.

#### Experiment 6:

To demonstrate the efficiency of shorter-length peptides which collectively are members of the PR-39 derived oligopeptide family in stimulating angiogenesis in-vivo, a novel peptide, PR11, composed of the first 11 amino acid residues

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[N-terminal end] of the native PR-39 sequence was purposely synthesized. The amino acid sequence of PR11 is as follows:

	1	2	3	4	5	6	7	8	9	10	11
	Arg	Arg	Arg	Pro	Arg	Pro	Pro	Tyr	Leu	Pro	Arg

To introduce the short-length PR11 peptide in-vivo, a mouse Matrigel assay system was utilized. In sum, either 5  $\mu$ g/ml of PR11 peptide or 5  $\mu$ g/ml of native PR-39 peptide were individually placed into a growth factor-depleted Matrigel pellet; and then each prepared Matrigel pellet was inserted into the peritoneal cavity of a mouse. After 14 days intraperitoneal placement, each pellet was removed from its living host; and each pellet was examined for evidence of new vascularity. The results are graphically presented by Fig. 7. Note that the bar graph of Fig. 7 shows the number of blood vessels [mean $\pm$ SD] per 10 high power fields (HPF).

As evidenced by Fig. 7, the analysis of Matrigel pellet vascularity after 14 days incubation in-vivo demonstrated significant induction of angiogenesis in both the PR11 and the native PR-39 pellets. The control Matrigel pellets, however, showed no evidence of angiogenesis as such. Clearly therefore, the short-length PR11 peptide is fully efficacious and effective in stimulating angiogenesis in-vivo.

#### Conclusions:

(1) The described experiments and empirical data have demonstrated that PR-39 peptide has the ability to selectively alter activity of 20S proteasome in human endothelial cells by interacting with proteasomes in a reversible manner. This interaction leads to suppression of I $\kappa$ B and HIF-1 $\alpha$  degradation while not affecting expression of other proteasome-dependent proteins such as p105 NF $\kappa$ B or HSP70. Unlike other proteasome inhibitors, treatment with PR39 is not associated with any cellular cytotoxicity. Thus, PR39 and its related peptides provide a unique and unforeseen means of regulating cellular function and stimulating angiogenesis.



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(2) Several observations also set PR39 apart from the conventionally known proteasome inhibitors. First, PR39-mediated inhibition of I $\kappa$ B $\alpha$  degradation is demonstrably reversible, unlike that of lactacystin. Second, long-term exposure of several cell types to PR39 did not result in any cytotoxicity, in contrast to the rapid cell death typically observed following cell treatment with lactacystin or MG132. This observation shows that PR39 peptide differentially affects processing of various and different intracellular proteins. Also supporting this view is the observation that while increasing HIF-1 $\alpha$  expression, PR39 administration had no meaningful effect on the expression of either NF $\kappa$ B or HSP70. Third, PR-39 peptide modulation of proteasome activity plays a functional role since the observed increased expression of HIF-1 $\alpha$  was directly associated with an increased expression of its target genes, VEGF and *flt-1*.

The present invention is not to be limited in form nor restricted in scope except by the claims appended hereto.

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What we claim is:

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1. A method for stimulating angiogenesis within a targeted collection of viable cells in-situ, said method comprising the steps of:

5 identifying a collection of cells comprising viable cells in-situ as a target for stimulation of angiogenesis;

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providing means for effecting an introduction of at least one member selected from the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells;

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10 introducing at least one member of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells using said effecting means;

allowing said introduced PR-39 oligopeptide collective member to interact with such proteasomes as are present within the cytoplasm of said targeted collection of cells whereby

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15 (a) at least some of the proteasomes interact with said PR-39 oligopeptide collective member, and

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(b) at least a part of the proteolytic activity mediated by said interacting proteasomes becomes selectively altered, and

20 (c) the selectively altered proteolytic activity of said interacting proteasomes results in a stimulation of angiogenesis in-situ within the targeted collection of viable cells.

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2. A method for selective inhibition of proteasome-mediated degradation of peptides in-situ within a collection of viable cells, said method comprising the steps of:

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identifying a collection of cells comprising viable cells in-situ as a target;

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providing means for effecting an introduction of at least one member selected from the group consisting of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells;

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introducing at least one member of the PR-39 oligopeptide collective to the cytoplasm of said targeted collection of cells using said effecting means;

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5 allowing said introduced PR-39 oligopeptide collective member to interact  
with such proteasomes as are present within the cytoplasm of said targeted  
collection of cells whereby

- 10 (a) at least some of the proteasomes interact with the PR-39  
5 oligopeptide collective member, and  
(b) at least a part of the proteolytic activity mediated by said  
15 interacting proteasomes becomes markedly altered, and  
(c) the markedly altered proteolytic activity of said interacting  
proteasomes results in a selective inhibition of proteasome-mediated degradation of  
10 peptides in-situ within the targeted collection of cells.

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3. The method as recited in claim 1 or 2 wherein said collection of viable cells  
includes at least one type of cell selected from the group consisting of endothelial  
25 cells, myocytes and myoblasts, fibrocytes and fibroblasts, epithelial cells,  
15 osteocytes and osteoblasts, neuronal cells and glial cells, erythrocytes, leukocytes,  
and progenitor cells of all types.

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4. The method as recited in claim 1 or 2 wherein said collection of cells  
comprises at least one tissue selected from the group consisting of myocardium,  
20 skeletal muscle, smooth muscle, an artery, a vein, lung, brain, kidney, spleen,  
35 liver, gastrointestinal tissue, nerve tissue, limbs, and extremities.

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5. The method as recited in claim 1 or 2 wherein the means for an  
25 introduction of a PR-39 oligopeptide collective member include one selected from  
the group consisting of catheter-based introduction means, injection-based  
introduction means, infusion-based introduction means, localized intravascular  
introduction means, liposome-based introduction means, receptor-specific peptide  
45 introduction means, slow releasing means for peptide secretion in living cells and  
sequestered organisms.

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6. The method as recited in claim 1 or 2 wherein the means for an  
50 introduction of a PR-39 oligopeptide collective member includes the DNA

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sequences coding for PR-39 oligopeptides of different sizes inserted in a suitable vector for transfection and subsequent expression of peptides within said cells.

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7. The method as recited in claim 1 or 2 wherein said method is practiced under in-vivo conditions.

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8. The method as recited in claim 1 or 2 wherein said method is practiced under in-vitro conditions.

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9. The method as recited in claim 1 or 2 wherein degradation of I $\kappa$ B $\alpha$  is selectively inhibited.

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10. The method as recited in claim 1 or 2 wherein degradation of HIF-1 $\alpha$  is selectively inhibited.

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11. A family of PR-39 derived oligopeptides whose members individually cause a selective inhibition of proteasome-mediated degradation of peptides in-situ after introduction intracellularly to a viable cell, each member of said oligopeptide family being:

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a peptide less than 39 amino acid residues in length;  
at least partially homologous with the N-terminal amino acid residue sequence of the native PR-39 peptide;  
able to interact in-situ with such proteasomes as are present within the cytoplasm of the cell; and

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able to alter markedly the proteolytic activity of said interacting proteasomes such that a selective increased expression of specific peptides occurs in-situ.

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12. The PR-39 derived oligopeptide family as recited in claim 11 whose membership includes a peptide comprised of 15 amino acid residues whose sequence is Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg-Pro-Arg-Pro-Pro.

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13. The PR-39 derived oligopeptide family as recited in claim 11 whose membership includes a peptide comprised of 11 amino acid residues whose sequence is Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr-Leu-Pro-Arg.

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5 14. The PR-39 derived oligopeptide family as recited in claim 11 whose membership includes a peptide comprised of 8 amino acid residues whose sequence is Arg-Arg-Arg-Pro-Arg-Pro-Pro-Tyr.

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House HSSIGTGYDL SASTFSPDGR VFOVEYAMKA VENSSTAIGI RCKDGVVIGV  
 Human -----  
 EKLVLKLYE EGSNKRLENV DRHVGHAVAG LLADARSLAD IAREEASNFR  
 -----  
 SNFGYNIPLK HLADRVANYV HAYTLYSVR PFGCSFHLGS YSANDGAOLY  
 -----  
 MIDPSGVSYG YNGCAIGKAR QAAKTEIEKL QMKDHTCRDV VKEVAKIYYI  
 -----  
 VHDEVKDAF ELELSWVGEL TKGRMEIVPK DIREEAKEYA KESLKEEDES  
 -----  
 DDDNM  
 -----

Fig. 1A

	C terminal tails	Net Charge
$\alpha 1$	AERD	-1
$\alpha 2$	A	0
$\alpha 3$	KKHEEEAKAEREKKEQKEKDK	+1
$\alpha 4$	EKEKEENEKKQKKAS	+2
$\alpha 5$		0
$\alpha 6$	EERPORKAQAQPADEPAEKADPEMH	-3
$\alpha 7$	AKESLKEEDESDDNM	-6

Fig. 1B

$\alpha 7$ subunit	Growth/LacZ+
1 _____ 255	+++
210 _____ 255	-
172 _____ 255	-
1 _____ 171	-

Fig. 1C

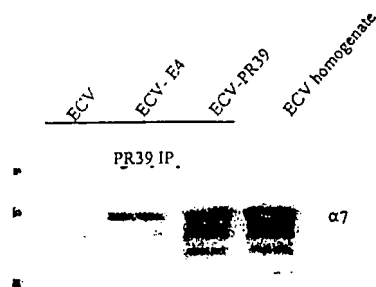


Fig. 1D

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Fig. 2A

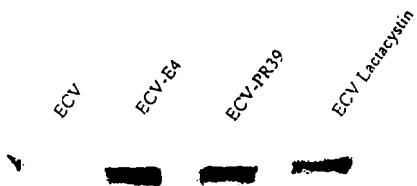


Fig. 2B

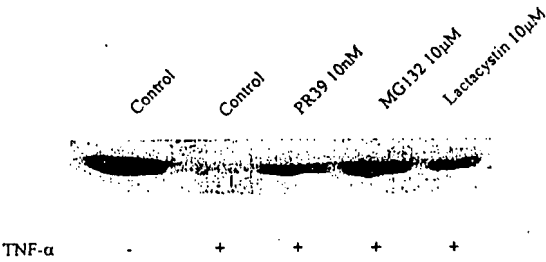


Fig. 2C

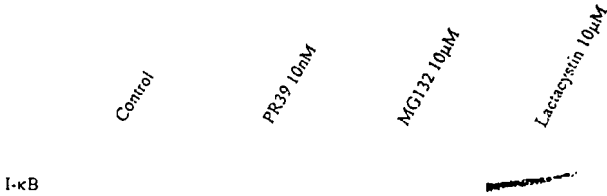


Fig. 2D

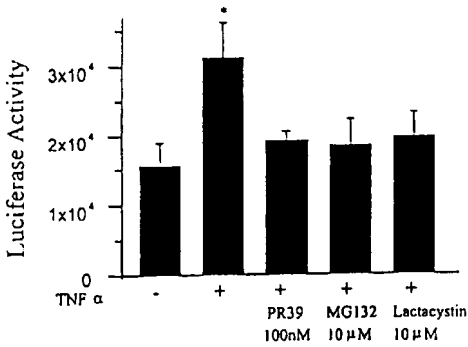


Fig. 3A

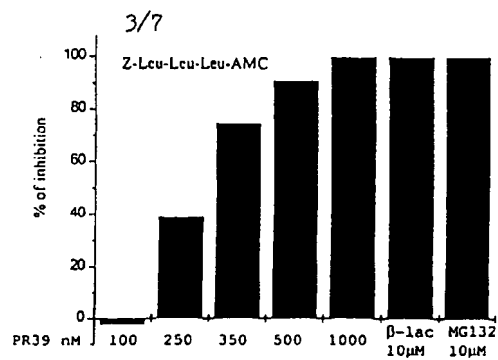
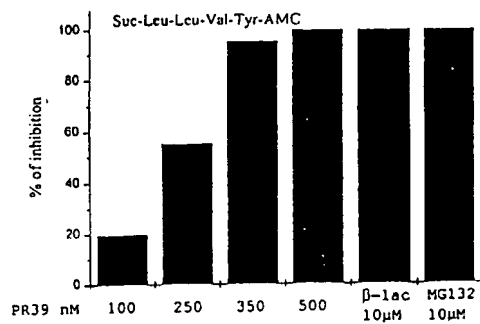


Fig. 3B





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Fig. 3C

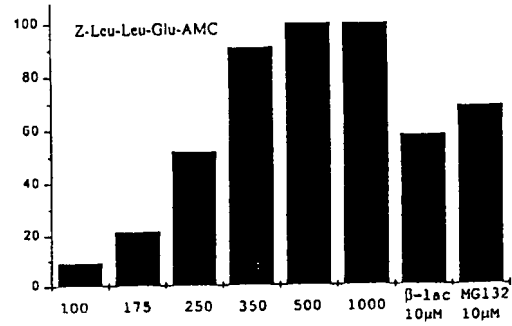
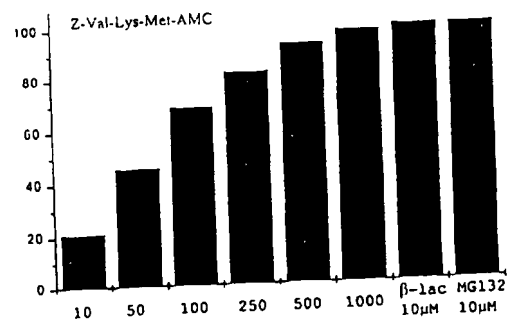
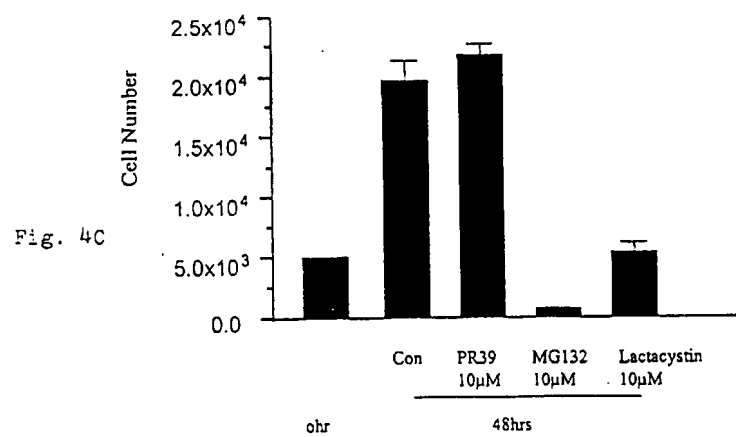
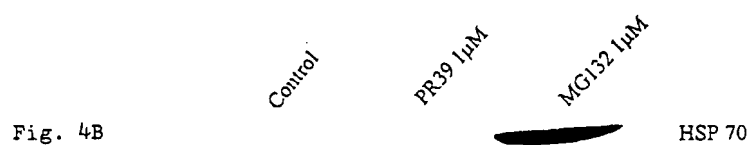
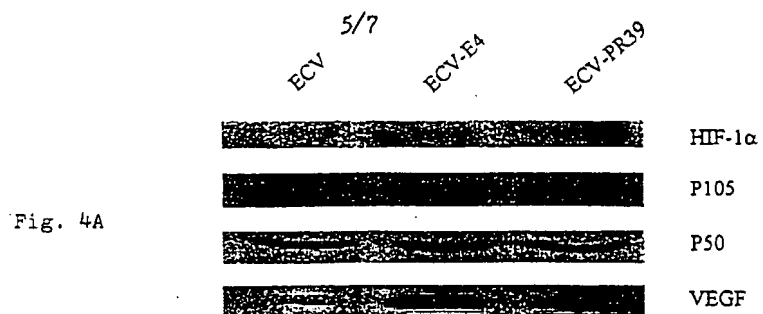


Fig. 3D





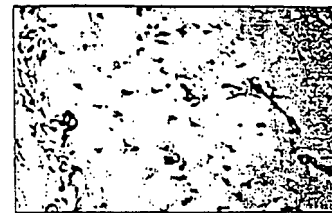
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Fig. 5A

Control



Fig. 5B



PR 39

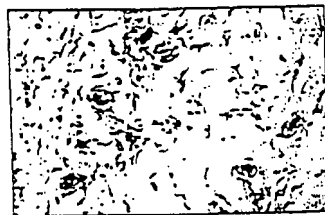
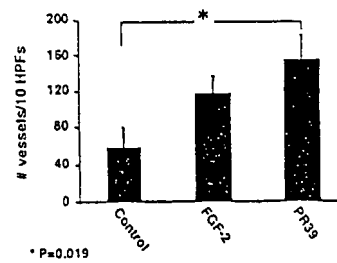


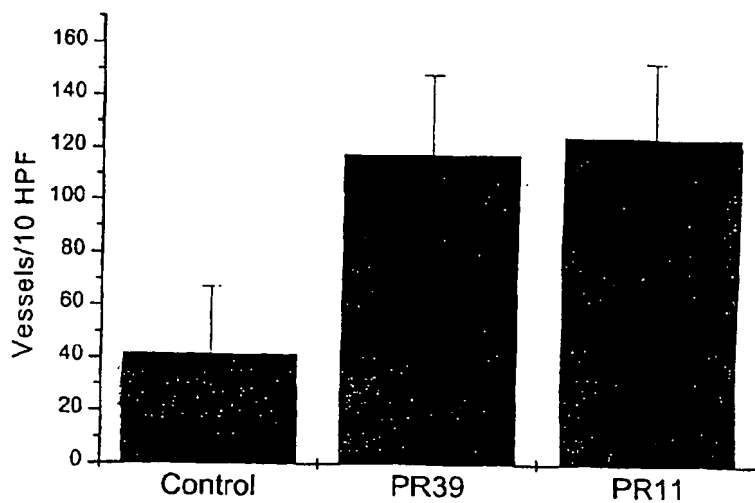
Fig. 5C

Fig. 6



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Fig. 7



## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US00/07050

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 38/00, 38/08, 38/16; C07K 7/00, 7/04, 7/06

US CL : 514/12, 15; 530/324, 327, 328

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/12, 15; 530/324, 327, 328

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAPLUS, MEDLINE, BIOSIS, USPATFULL

search terms: polypeptide sequences, angiogenesis, inhibit, stimulate, proteasome

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96/09322 A2 (CHILDREN'S MEDICAL CENTER CORPORATION) 28 March 1996, see especially the abstract, pages 10-12 and page 24, lines 1-14.	1-14
Y	LI, JIAN et al. "Cardiac-specific overexpression of PR-39 induces angiogenesis, myocardial hypertrophy, and increased microvascular reactivity." Circulation, 27 October 1998, Vol. 98, No. 17, SUPPL., page 1794, Abstract No. 4163, see entire document.	1-14

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Z" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

01 JUNE 2000

Date of mailing of the international search report

04 AUG 2000

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